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Supplemental Data

Rumi Is a CAP10 Domain Glycosyltransferase that Modifies Notch and Is Required for Notch Signaling

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Supplemental Experimental Procedures

Genetics

rumi mutant embryos

To obtain *rumi* embryos that lack both maternal and zygotic contribution, $rumi^{\Delta 26}/^{\Delta 26}$ flies were crossed to each other at 18°C and once the animals started to lay eggs they were transferred to 28°C.

Clonal analysis, MARCM, modified MARCM

Mitotic clones were generated by using either *Ubx-FLP* or a combination of *C684-GAL4* and *UAS-FLP*. For MARCM clones, animals were raised at room temperature until the second instar larval period and were then transferred to 28°C. The following males were crossed to *Ubx-FLP tub-GAL4 UAS-GFP^{nls}-6X-Myc; FRT82B y^+ tub-GAL80/TM6, Ubx* females to obtain flies with MARCM clones: *y w; FRT82B $rumi^{\Delta 26}$/TM6, Tb*, *y w; UAS-N^{FL}; FRT82B $rumi^{\Delta 26}$/SM5-TM6*, *y w; UAS-N^{FL}/+; FRT82B/+*, *y w; UAS-N^{ECN}; FRT82B $rumi^{\Delta 26}$/SM5-TM6*, *y w; UAS-Dl^{30}/+; FRT82B $rumi^{\Delta 26}$/+*, *y w; UAS-Ser/+; FRT82B $rumi^{\Delta 26}$/+*. Overexpression of Delta and Serrate was confirmed by antibody staining. For the modified MARCM experiments, *y w Ubx-FLP Tub-GAL4 UAS-GFP^{NLS}-6X-Myc; FRT82B* females were crossed to males with *y w; UAS-Dl^{30}/CyO; FRT82B $rumi^{\Delta 26}$ y^+ tub-GAL80/+* or *y w; UAS-Ser/CyO; FRT82B $rumi^{\Delta 26}$ y^+ tub-GAL80/D* genotypes. $rumi^{\Delta 26}$ clones were detected based on Notch accumulation. Wing overgrowth and the presence of multiple regions of aberrant Cut expression were used to select wing discs with Ser and Dl overexpression.

In vivo role of the KDEL motif

To assess the ability of low-to-moderate levels of Rumi-FLAG and Rumi-FLAG-ΔKDEL in rescuing *rumi* clones, *y w; UAS-FLP; C684-GAL4 FRT82B $rumi^{44}$/TM6, Tb* females were crossed to *y w; UAS-rumi-FLAG/L; FRT82B πM Sb^{63} ry^+ y^+/+* (two independent insertions) or *y w; UAS-rumi-FLAG-ΔKDEL/L; FRT82B πM Sb^{63} ry^+ y^+/+* (three independent insertions) and raised at 28°C. L^+ animals with mitotic clones were scored

for the presence of y^{-} Sb^{+} bristles. The ability of higher levels of Rumi-FLAG and Rumi-FLAG-ΔKDEL in rescuing *rumi* clones was assessed in flies with the following genotypes: *y w Ubx-FLP tub-GAL4 UAS-GFP^{nls}-6X-Myc; UAS-rumi-FLAG/+; FRT82B y^{+} tub-GAL80/FRT82B rumi$^{\Delta 26}$* (or *UAS-rumi-FLAG*-ΔKDEL /+).

<u>*Notch* transcription in *rumi* clones</u>

Notch-lacZ/+; FRT82B rumi$^{\Delta 26}$/+ females were crossed to *y w Ubx-FLP tub-GAL4 UAS-GFP^{nls}-6X-Myc; FRT82B y^{+} tub-GAL80/TM6, Ubx* males. Female progeny of this cross with MARCM clones were selected under a GFP scope and stained with anti-Notch and anti-βGAL antibodies to ensure the presence of *rumi$^{\Delta 26}$* and *Notch-lacZ*, respectively.

<u>Genetic interaction studies</u>

To examine the dosage effect of *Notch* on the *rumi^{44}* phenotype, *Notch^{55e11}/+; rumi^{44}/+* females were crossed to *rumi^{44}/TM6, Tb* males and raised at the designated temperature. Since loss of one copy of *rumi* does not affect the *Notch* haploinsufficient phenotype (data not shown), correct genotypes were identified based on the wing and bristle phenotypes. Similar results were obtained by using *Df(1)Notch^{8}*, which uncovers the *Notch* locus, or *w; Df(3R)Exel6192/TM6, Tb,* which uncovers the *rumi* locus. Also, *SM1, Dp(1;2)51b/ +; rumi^{44}/+* males were crossed to *rumi^{44}/TM6, Tb* females, and the *Curly* phenotype was used to follow the *SM1, Dp(1;2)51b* chromosome. A similar crossing scheme was used to asses the effects of increasing *Notch* dosage on the *rumi$^{\Delta 26}$/Df(3R)Exel6192* phenotype presented in Figure S1. For S1A and S1B, the animals were kept at 23°C throughout the development. For S1C and S1D, the larvae were kept at 18°C until late third instar. They were then transferred to 28-30°C for 24-28 hours. The larvae were kept at 18°C for the remainder of development.

To test the dosage effect of *Delta* on the *rumi* phenotype, Sb^{+} Tb^{+} progeny of the cross between *Dl^{9P} rumi^{44}/TM3, Sb^{1}* and *rumi^{44}/TM6, Tb* flies were scored for wing, eye and leg phenotypes.

To examin the genetic interaction between *lgd* and *rumi*, progeny of the following crosses were used:

Figure S8A-B': *y w Ubx-FLP; lgd^{1} FRT40A; FRT82B rumi$^{\Delta 26}$/SM5-TM6* females were crossed to *ubi-GFP FRT40A/CyO; FRT82B πM Sb^{63} ry^{+} y^{+}/TM6* males.

Figure S8C-C"': *y w Ubx-FLP; lgd^{1} FRT40A; FRT82B rumi$^{\Delta 26}$/SM5-TM6* females were crossed to *hs-FLP; lgd^{1} FRT40A/CyO; FRT82B ubi-GFP/TM3, Sb^{1}* males. Selection of *lgd$^{1/1}$* larvae was facilitated by their significantly enlarged wing discs.

Antibody production and immunostainings

The region of *rumi* open reading frame (ORF) that encodes the first 150 amino acids was cloned into the *pGEX-4T1* vector (GE Healthcare). The fusion protein was produced in bacteria and purified using glutathione sepharose 4B beads (GE Healthcare). The purified fusion protein was then run on a large SDS Polyacrylamide gel. The corresponding band was cut and sent to Cocalico Biologicals (Reamstown, PA) for antibody production.

Dissections and stainings were performed by using standard methods. For surface staining of Notch in *rumi* clones, detergents were excluded from the staining protocol, as described previously (Wang and Struhl, 2004). Primary antibodies used in this study are rat α-Elav 1:500 (7E8A10; DSHB), mouse α-Cut 1:500 (2B10; DSHB), rat α-DE-Cad 1:1000 (DCAD2; DSHB), guinea pig α-Boca 1:1000 (Culi and Mann, 2003), guinea-pig α-Dl 1:3000 (M. Muskavitch), mouse α-Dl^{ECD} 1:1000 (C54.9B; DSHB), mouse α-N^{ICD} 1:1000 (C17.9C6; DSHB), guinea-pig α-HSC3 1:1000 (Ryoo et al., 2007), mouse α-N^{ECD} 1:100 (C458.2H; DSHB), rabbit α-βGAL 1:1000 (Cappel), rat α-Serrate 1:2000 (Panin et al., 1997), mouse α-Wingless 1:10 (4D4; DSHB), rabbit α-FLAG 1:300 (Invitrogen) and guinea pig α-Rumi 1:2000 (this study). Secondary antibodies were used at 1:500 and are Alexa488-conjugated (Molecular Probes), and Cy2-, Cy3- and Cy5-conjugated (Jackson ImmunoResearch Laboratories).

Image acquisition and processing

Confocal images were scanned by using an LSM510 confocal microscope and processed with Amira3.1 (Indeed, Berlin, Germany) and Adobe Photoshop 7.0. For adult thorax images, animals were boiled in 10% KOH, dissected, and mounted in 70% glycerol in PBS. A Zeiss AxioCam MRm camera mounted on an LSM10 confocal microscope was used to obtain stacks of images. The Extended Focus function of the AxioVision software (Zeiss) was used to obtain the final images. Adult wings and legs were incubated in 100% ethanol for 2-3 minutes, dried and mounted in the DPX medium (Electron Microscopy Sciences). Sample preparation and image acquisition for scanning electron microscopy were essentially performed as described previously (Frankfort et al., 2004). Final figures were assembled in Adobe Illustrator CS2.

Mapping of the imprecise *P*-element excision based deletion lines

To characterize the molecular nature of the *EY00249* excisions, we used two approaches: (1) we designed primer pairs to generate overlapping ~600 bp PCR products covering several kbs on each side of the *P*-element insertion site, and used the primers to amplify each fragment from genomic DNA isolated from animals homozygous for each of the imprecise excisions with *rumi* phenotype and also from *y w* and the original *EY00249* line as control. (2) we used PCR primers to bridge the gap generated in the genome via imprecise excision of the *EY00249*. In most cases, we did not obtain a PCR product, strongly suggesting that parts of the *P*-element still remain in the locus. However, we obtained a PCR fragment from excision *Δ26*, sequencing of which showed that the *P*-element is completely excised, along with 95% of the *rumi* coding region.

Molecular Biology

rumi ORF was generated by PCR using first strand embryonic cDNA, and cloned into the *pUAST* vector. A 3' primer containing the DNA sequence that encodes a FLAG tag peptide and a stop codon was used to amplify *rumi* ORF to obtain *rumi-FLAG-ΔKDEL* insert. A second PCR using a primer that encodes a KDEL and a stop codon was used to generate the *rumi-FLAG* insert. Both inserts were cloned into the *pUAST* vector.

To obtain Rumi genomic rescue construct, a 3kb genomic fragment that only contains the *rumi* gene was amplified by PCR and inserted into the *pBluescript-SK* vector. Using the

QuikChange Site-Directed Mutagenesis kit (Stratagene), a FLAG peptide was inserted before the KDEL motif. The original 3kb fragment and the 3kb fragment with the FLAG tag were then cloned into the *pCaSpeR-4* vector. All constructs were verified by sequencing.

The region that encodes the signal peptide of the *Drosophila* Acetylcholine esterase protein (*CG17907*) was amplified by PCR and cloned into the *pMT/V5-HisB* vector (Invitrogen) in-frame with the V5 and His tags. A CAAC optimal translation start sequence was incorporated before the start codon ATG via the 5' PCR primer (*pMT/V5B-ACE*). The region that covers the 7th EGF repeat to the transmembrane domain of Notch (EGF7-TM) was amplified by PCR. The EGF7-TM fragment was inserted into *pMT/V5B-ACE* in-frame with the signal peptide and the C-terminal V5 and His tags. S2 cells that are adapted to serum free media (SFM, Invitrogen) were transfected with *pMT/V5B-EGF7-TM* using the FuGENE-HD transfection reagent (Roche). One day after transfection, S2 cells were divided into two equal groups. One was treated with dsRNA against *EGFP*, the other was with dsRNA against *rumi.* Two days after dsRNA treatment, expression of the *pMT/V5B-EGF7-TM* construct was induced using 0.7 mM $CuSO_4$. Three days after induction, media from both groups were collected after spinning down the cells at 300*g* for 10 minutes. The media were dialyzed three times using 1X binding buffer for His Bind Resin (Novagen). After dialysis, the His-tagged EGF7-TM protein secreted in the media was purified using His Bind Resin (Novagen) according to the manufacturer's protocol. After purification, the eluted protein was TCA precipitated and washed with cold acetone before mass spectral analysis.

Receptor ligand interaction and S2 cell culture assays

Receptor ligand interactions were performed as described previously (Bruckner et al., 2000; Childress et al., 2006). Shortly, we transfected S2 cells with a construct that expresses the Notch extracellular domain linked to alkaline phosphatase (N-AP) (Gift from K. Irvine and S. Cohen) with or without a construct that expresses Fringe-Myc (Gift from S. Cohen). The next day, these cells were then treated with either a dsRNA against *EGFP* (control) or a dsRNA against *rumi* (experiment) and kept at 28°C. Two days after dsRNA treatment, the cells were induced with 0.7 mM $CuSO_4$. The media from these cells were collected 3 days after induction, and the N-AP levels present in the media were equilibrated based on the alkaline phosphatase (AP) activity. The media containing N-AP and 0.1% sodium azide were then added to S2 cells that express Delta (S2-Dl). After a 90-minute incubation (at room temperature or at 28°C), S2-Dl cells were washed with regular media to remove N-AP that had failed to bind to the cells expressing Delta. To measure the AP levels, chemiluminescent AP reagent from BioFX and FLUOstar Optima Plate Reader from BMG Labtech were used.

Production of Rumi protein in S2 cells

Two groups of equal amounts of S2 cells were prepared. One group was transfected with *pUAST-rumi-FLAG* and *pAc-Gal4* and the other was transfected with *pUAST* and *pAc-Gal4* constructs (control). The amount of *pUAST-rumi-FLAG* and *pUAST*, and the amounts of *pAc-Gal4* transfected in both groups were the same. Three days after

transfection cells were collected by spinning at 300*g* for 10 minutes and the media were preserved. Cells were washed with 2X PBS and lysed with CelLytic-M reagent (SIGMA) and protease inhibitors. After the lysis, insoluble fragment was removed by centrifugation. Immunoprecipitation (IP) was performed using media and soluble fragment of the cell lysates from both groups. EZview Red ANTI-FLAG M2 affinity gel (SIGMA) was used for the IP reaction according to the manufacturer's protocol.

Protein *O*-glucosyltransferase assay

O-glucosyltransferase assays were performed with slight modification as previously described (Shao et al., 2002). Briefly, the standard 10 μl reaction mixture contained 50 mM HEPES pH 7.0, 10 mM $MnCl_2$, the indicated amounts of factor VII EGF, 0.16 μM (0.01 μCi/μl) UDP-[^{3}H]glucose (American Radiolabeled Chemicals, Inc), 10 μM UDP-glucose, 0.5% NP-40, and 6 ng of the purified Rumi protein. The reaction was incubated at 37°C for 20 minutes and stopped by adding 900 μl of 100 mM EDTA pH 8.0. The sample was loaded onto a C18 cartridge (100 mg). After washing with 5 ml of H_2O, the factor VII EGF was eluted with 1 ml of 80% methanol. Incorporation of [^{3}H]glucose into the factor VII EGF was determined by scintillation counting of the eluate. Reactions without substrates were used as background control.

Western blot analyses and RNAi for Notch cleavage assays

Extract preparation and western blots were performed as described previously (Hu et al., 2002; Pan and Rubin, 1997; Ye et al., 1999). For Kuzbanian RNAi in S2 cells, three different dsRNAs were used. *kuz-A* refers to the dsRNA used in Figure 8A in (Lieber et al., 2002). *kuz-HFA*, *kuz-BKN* and *rumi-HFA* dsRNAs were designed using Flybase. Three days after treatment with the corresponding dsRNA, S2 cells were transfected with equal amounts of *pMT-Notch* construct. One day after transfection, Notch expression was induced with 0.7 mM CuSO4 for four hours. S2 cells were then lysed in a hypotonic solution as described previously (Hu et al., 2002). Decanoyl-RVKR-CMK (Emd Biosciences) was used as the furin inhibitor at a final concentration of 50 μM and was added one hour before induction. S2 cells were kept at 28°C throughout the experiment.

Supplemental Results

ER retention is required for the function of Rumi

Rumi contains a signal peptide and a KDEL motif found in soluble ER-retained proteins (Munro and Pelham, 1987). Indeed, a Myc-tagged version of the human Rumi homolog localizes to the ER when overexpressed in COS-7 cells (Teng et al., 2006). To determine the importance of KDEL, we expressed FLAG-tagged versions of wild-type Rumi and Rumi-ΔKDEL in S2 cells (Figure S4A, RF and RFΔKDEL). As shown in Figure S4, endogenous Rumi is present in S2 cell extracts, but not in the medium. Overexpression of Rumi leads to a significant increase in Rumi protein, some of which is secreted into the medium. This suggests that Rumi is a soluble protein that cannot be fully retained in the ER when overexpressed. Indeed, expression of RFΔKDEL results in less retention in cells and increased levels in the medium (Figure S4B). Hence, Rumi is a soluble protein that requires the KDEL motif for efficient ER retention. We then generated transgenic

lines to express RF and RFΔKDEL in flies. Low- to moderate-level expression of RF fully rescues the bristle phenotype of *rumi* mutant clones raised at 25ºC (Figure S4D versus S4C). However, RFΔKDEL is not able to rescue the *rumi* bristle phenotype when expressed at similar levels (Figure S4E). We therefore conclude that ER retention is required for the proper function of Rumi. Note that high-level expression of both RF and RFΔKDEL in MARCM clones of *rumi* rescues the bristle phenotype (Figures S4F and S4G). This indicates that if at each point in time enough newly-made Rumi is present in the ER, the KDEL-mediated retrograde transport of Rumi from the Golgi to ER becomes dispensable.

The *O*-glucosyltransferase activity mediated by Rumi is required for proper Notch signaling

DXD-like motifs have been implicated as catalytic sites in several glycosyltransferases (Wiggins and Munro, 1998). We identified a DXD-like motif in Rumi (ERD 236-238) and mutated it to ERA and ARA. The ERA mutant has 10% and the ARA mutant has 4% of the wild-type *O*-glucosyltransferase activity, and both are expressed and secreted as wild-type Rumi (data not shown). To assay their activity in vivo, we created transgenic flies. When these mutants were overexpressed in *rumi* mutants we observed a remarkable correlation between the level of *O*-glucosyltransferase activity and rescue of the *rumi* phenotype. Transgenic overexpression of the ARA mutant (4% *O*-glucosyltransferase activity) failed to rescue *rumi* in three independent transgenic strains, and provided a very weak rescue in one transgenic strain. The ERA (10% activity) mutant only partially rescued in four transgenic lines examined (one transgene rescued reasonably well, two exhibited only a partial rescue, whereas one transgene did not rescue the *rumi* phenotype). In contrast, all wild-type *rumi* transgenic strains (4 out of 4) rescued the null mutant phenotype fully (data not shown). These data provide strong evidence that *O*-glucosylation is the key function of Rumi.

Supplemental figures

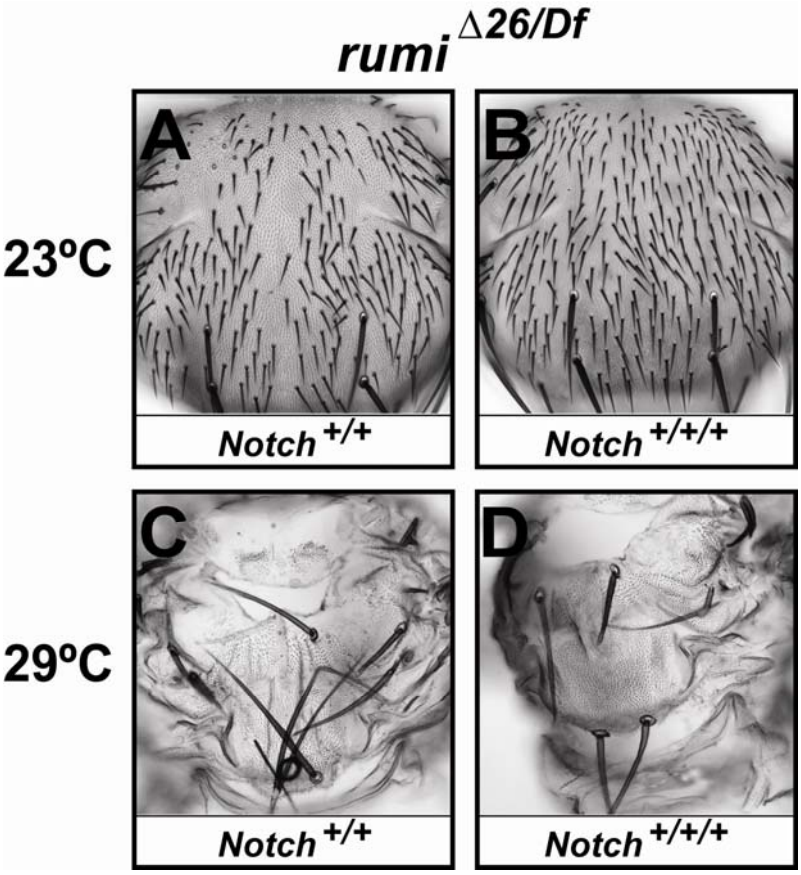

Figure S1. Addition of *Notch* gene dosage is not able to rescue the *rumi* phenotype at 28-30°C

(A) *rumi*$^{\Delta 26/Df}$ flies lose some of their microchaetae when raised at 23°C.

(B) Addition of an extra copy of *Notch* rescues the bristle loss in *rumi*$^{\Delta 26/Df}$ flies at 23°C.

(C) *rumi*$^{\Delta 26/Df}$ flies lose all of their microchaetae when kept at 28-30°C during microchaetae development.

(D) Addition of an extra copy of *Notch* does not rescue the microchaetae loss at 28-30°C in *rumi*$^{\Delta 26/Df}$ flies.

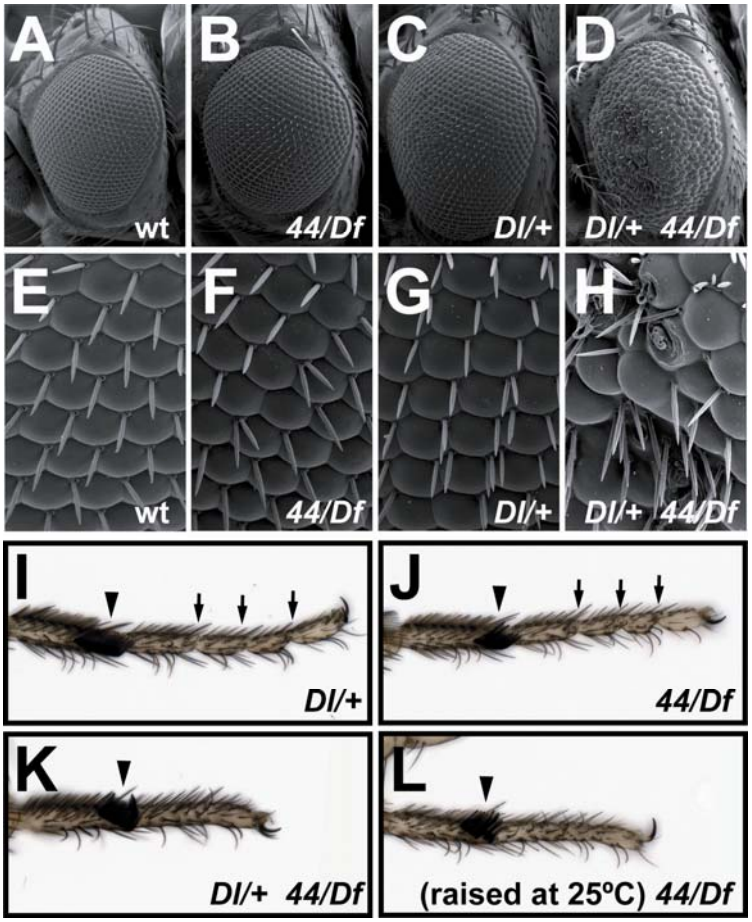

Figure S2. *rumi* genetically interacts with *Delta* in several tissues

(A-H) Genetic interaction between *rumi* and *Delta* in the eye. Scanning electron micrographs of 2-3 day old flies are shown. (E-H) are close-ups of (A-D) respectively. All flies are raised at 18°C.

(A and E) The eye of a wt fly.

(B and F) The eye of a $rumi^{44}$ hemizygous fly is smooth. Note the presence of occasional extra inter-ommatidial bristles.

(C and G) The eyes of *Delta* heterozygous flies also bear some extra inter-ommatidial bristles but are smooth.

(D and H) Flies hemizygous *for* $rumi^{44}$ and heterozygous for *Delta* show a severe rough eye phenotype and have numerous extra inter-ommatidial bristles and severe structural defects.

(I-L) Genetic interaction between *rumi* and *Delta* in the leg. Flies were raised at 18°C, except for the animal shown in panel L, which was raised at 25°C. Arrowhead depicts the sex comb, arrows show the leg joints.

(I) Flies that are heterozygous for *Delta* do not display an abnormal leg phenotype.

(J) $rumi^{44}/Df$ flies do not show an abnormal leg phenotype.

(K) Flies that are heterozygous for *Delta* and hemizygous for $rumi^{44}$ display segment fusion and leg shortening phenotypes, very similar to the phenotype observed in $rumi^{44}$ hemizygous flies raised at 25°C (L).

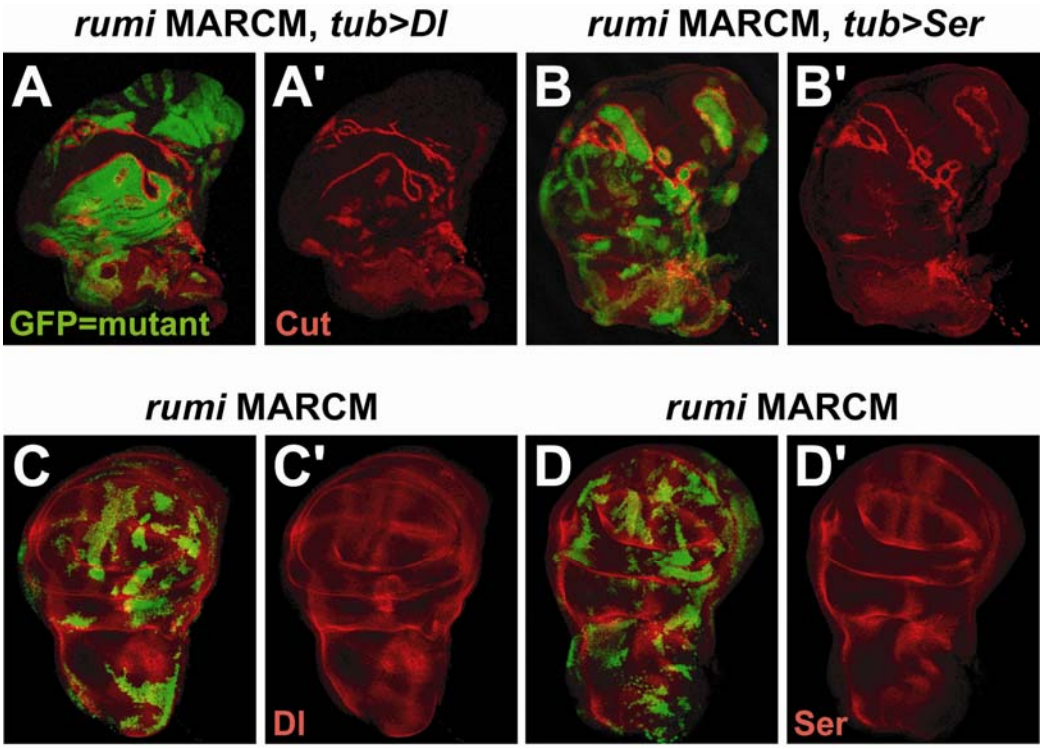

Figure S3. *rumi* is not required in the signal-sending cell
(A-B') Ectopic expression of *Delta* (A and A') or *Serrate* (B and B') in MARCM clones of *rumi* raised at 28-30°C induces robust Cut (red) expression in adjacent cells. GFP (green) marks the cells that are mutant for *rumi* and simultaneously overexpress *Delta* (A) or *Serrate* (B).
(C-D') Expression pattern of *Delta* (red in C and C') and *Serrate* (red in D and D') is not altered in *rumi* mutant clones raised at 28-30°C marked by GFP (green).

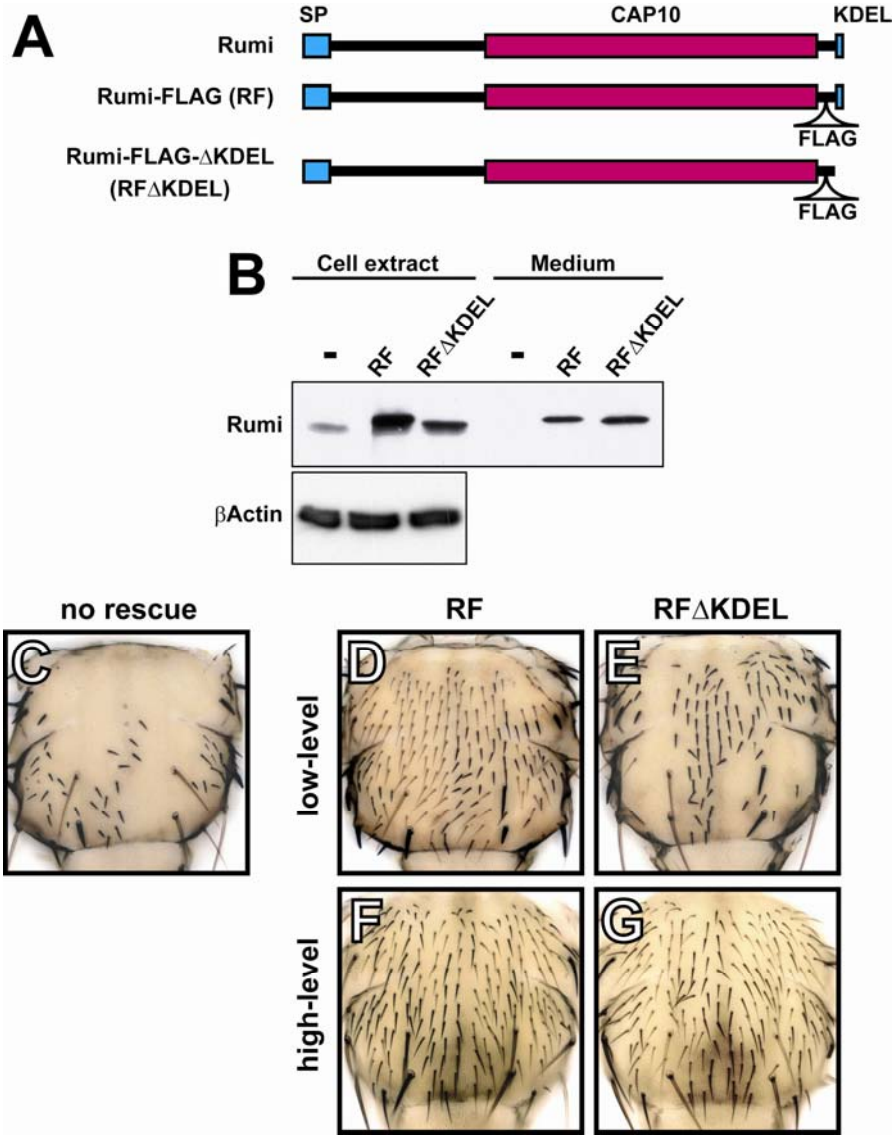

Figure S4. The KDEL ER-retention motif is required for proper Rumi function
(A) Schematic representation of wild-type Rumi protein (Rumi), along with FLAG-tagged versions of Rumi with or without the KDEL motif (RF and RFΔKDEL, respectively) that are used in (B-G).
(B) Western blots with a polyclonal α-Rumi antibody on cell extracts and media from S2 cells without any exogenous construct or transfected with RF or RFΔKDEL vectors. Over-expression of Rumi-FLAG in S2 cells causes secretion of the Rumi protein. Rumi with a KDEL motif (RF) is retained by the S2 cells more than Rumi without a KDEL motif (RFΔKDEL).
(C) Bristle development is impaired in *rumi* mutant clones at 25°C.
(D and E) Low- to Moderate-level expression of RF (D), but not RFΔKDEL (E), with the *C684-GAL4* driver can rescue the bristles in *rumi* mutant clones.
(F and G) High level expression of both RF and RFΔKDEL in MARCM clones of *rumi* rescues the bristle phenotype.

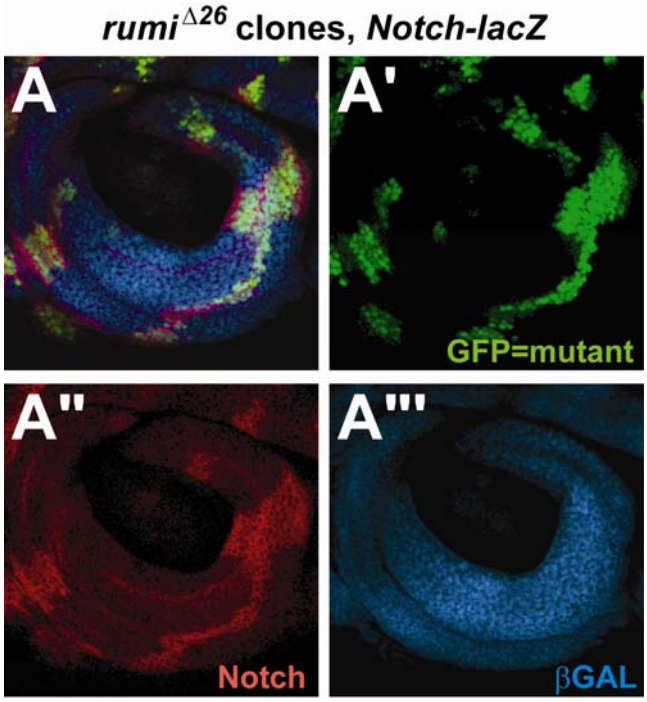

Figure S5. The *Notch* gene expression is not altered in *rumi* mutant cells
(A-A''') Shown is the wing pouch region of a third instar larva raised at 28°C. Although Notch protein levels (red) increase (A"), expression of βGAL (blue) from the *Notch-lacZ* transgene is not altered (A"') in *rumi* mutant clones (green).

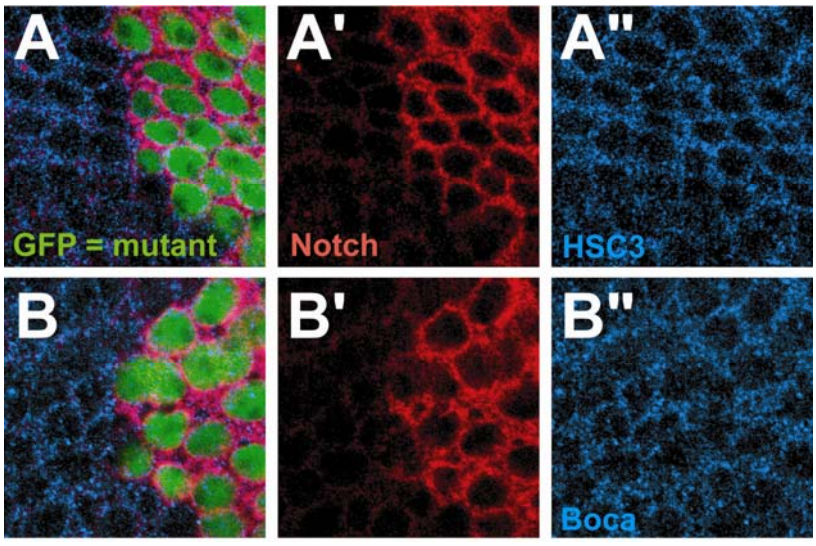

Figure S6. The UPR response is not activated in *rumi* mutant cells
(A-A'') Expression of HSC3 (blue), a chaperone induced by the unfolded protein response (UPR), does not increase in *rumi* cells at 28°C despite Notch accumulation (red).
(B-B'') Expression of Boca (blue), an ER localized protein, is not altered in *rumi* mutant cells (marked by the presence of the nuclear GFP), despite Notch accumulation (red).

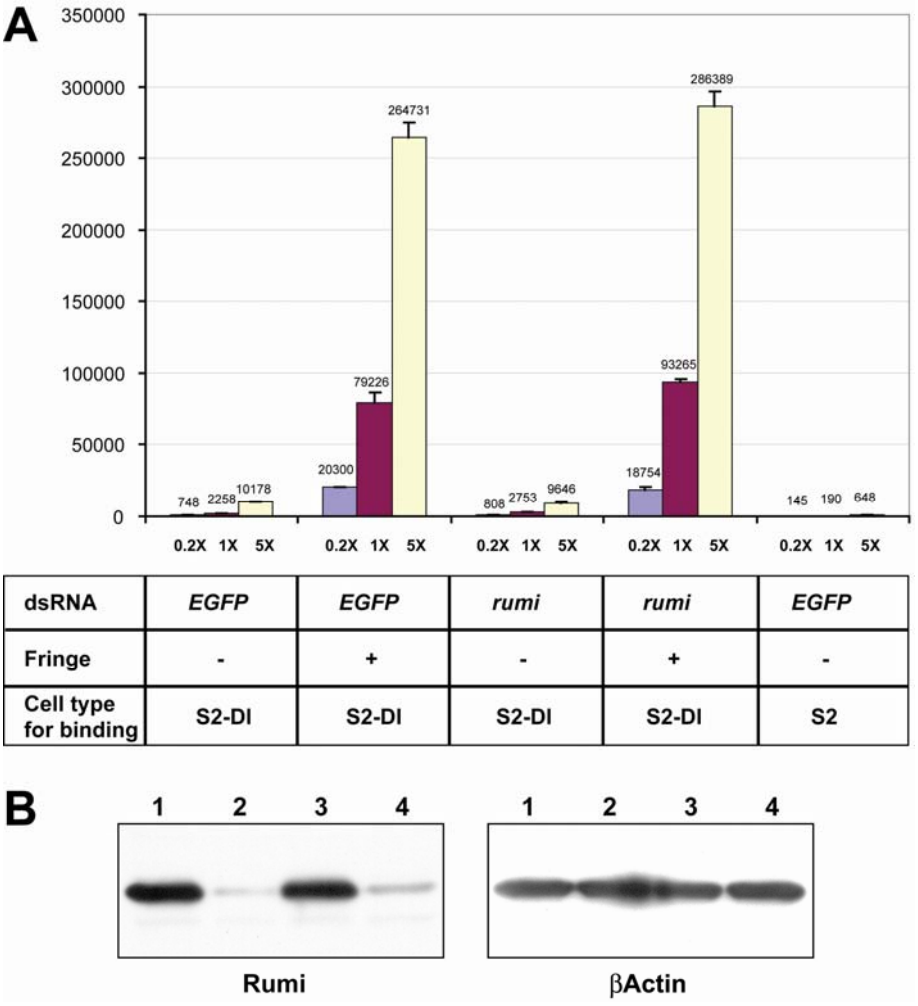

Figure S7. The binding between Notch and Delta is not decreased upon RNAi-mediated Rumi knockdown

Receptor-ligand interaction assays were performed at 28°C using different concentrations of N-AP. N-AP was produced as explained in Supplemental Experimental Procedures in S2 cells that were treated with dsRNA against *EGFP* or *rumi* in the presence and absence of Fringe. 0.2X, 1X and 5X refer to different concentrations of N-AP used in this experiment. Numbers on the bars show measured alkaline phosphatase activity (Y axis).

(A) Severe reduction of Rumi in S2 cells during N-AP expression does not alter the interaction between N-AP and S2-Dl cells at three different N-AP concentrations both in the absence and in the presence of Fringe. Error bars are standard error of the mean.

(B) Left gel: Western blot showing a significant reduction in Rumi protein levels in S2 cells that are treated with dsRNA against *rumi.* Lanes 1 and 3 are extracts from S2 cells that are treated with dsRNA against *EGFP*. Lanes 2 and 4 are extracts from S2 cells that are treated with dsRNA against *rumi*. Lanes 1 and 2 were transfected with *N-AP* expression construct. Lanes 3 and 4 were transfected with *N-AP* and *fringe-myc* expression constructs.

Right gel: Western blot using anti-βActin antibody for the samples used in the left gel.

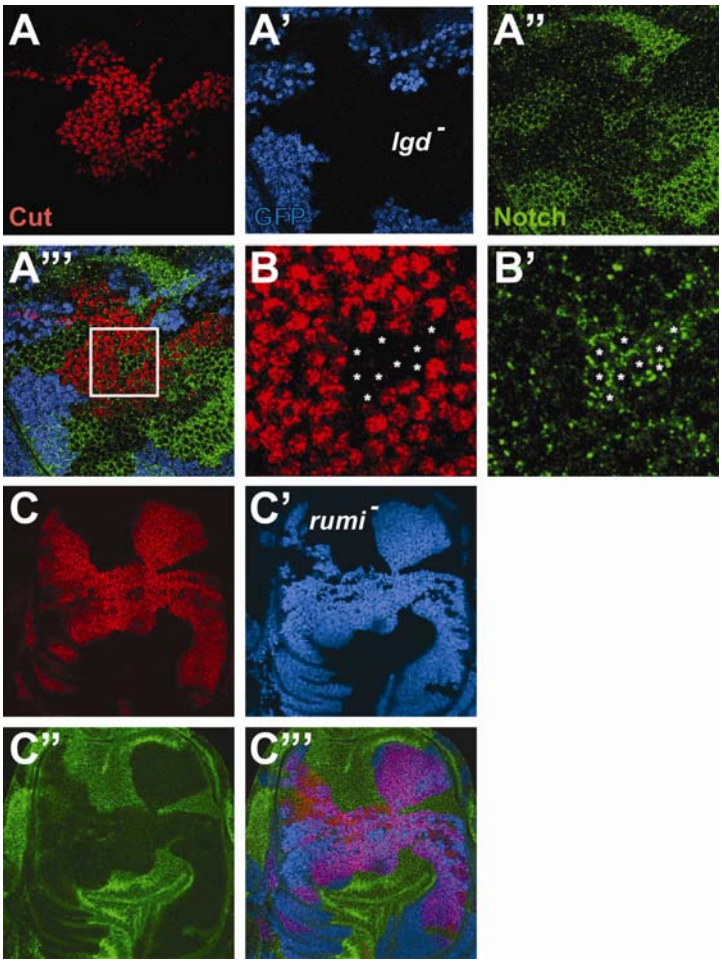

Figure S8. Ectopic activation of Notch signaling in *lgd* mutant cells is suppressed by loss of Rumi function

(A-A''') In large *lgd* mutant clones, Notch signaling is ectopically induced, causing ectopic expression of Cut (red) in cells close to the dorso-ventral boundary. *lgd* mutant clones are marked by the absence of GFP (blue). *rumi* mutant clones are marked by the increase in Notch protein levels (green). In cells that are double mutant for *lgd* and *rumi*, Notch protein levels increase, and Cut expression is lost at the restrictive temperature. The square in (A''') shows a region of *lgd* mutant cells that ectopically express Cut. Cells that are double mutant for *rumi* and *lgd* do not express Cut. (B and B') A close-up of the white square in (A'''). Asterisks mark the cells that are double mutant for *rumi* and *lgd*. Note that Notch protein levels significantly increase in *rumi* mutant cells regardless of the presence of Lgd. The double mutant cells do not express Cut.

(C-C''') The wing pouch region of a third instar larva which is homozygous mutant for *lgd*, harbors mitotic clones of *rumi* marked by the absence of GFP (blue) and is raised at 28°C. In animals that are homozygous mutant for *lgd*, Cut expression (red) in the wing margin is significantly broadened due to ectopic activation of Notch signaling. However, in cells that are mutant for both *rumi* and *lgd*, Cut expression is lost, similar to *rumi* mutant cells. Note the accumulation of Notch (green) in *rumi* mutant cells.

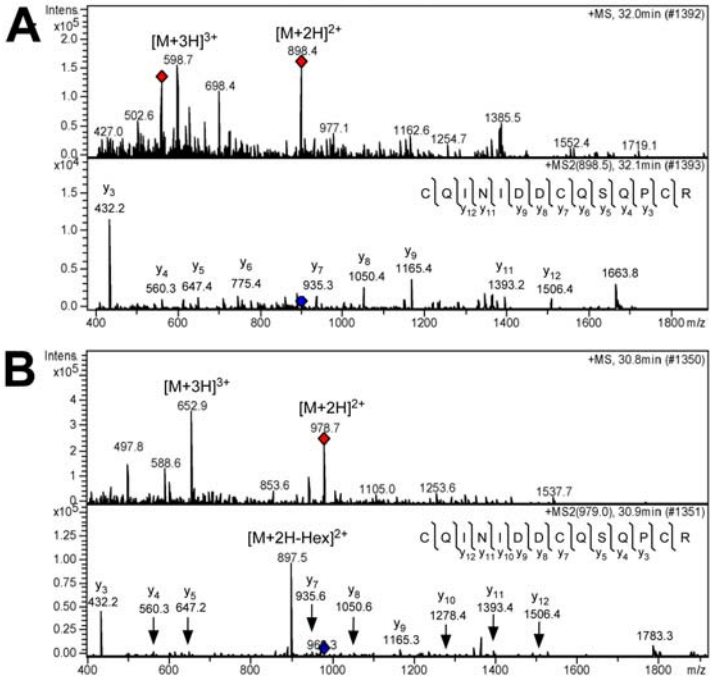

Figure S9. Knockdown of Rumi in S2 cells results in reduced O-glucosylation of a tryptic peptide from EGF repeat 14

Tryptic peptides generated from Notch EGF7-TM made in cells with or without Rumi were analyzed by LC-MS/MS as described in Experimental Procedures.

(A) Identification of the peptide ^{561}CQINIDDCQSQPCR^{574} from EGF repeat 14 in the Rumi knockdown sample. The top panel shows a full MS spectrum of material eluting at 32.0 min. The ions labeled $[M+2H]^{2+}$ and $[M+3H]^{3+}$ match the predicted mass for doubly and triply charged forms of the peptide, respectively. Other ions are from co-eluting material. CID fragmentation of the doubly charged form of this peptide, m/z 898.4 (top panel, $[M+2H]^{2+}$) resulted in the MS/MS spectrum shown in the bottom panel. Numerous sequence fragment ions (y-ions are shown) are observed that confirm the identity of the peptide. Ions selected for fragmentation in the MS spectrum are identified by red diamonds. The position of the parent ion fragmented in the MS/MS spectrum is identified with a blue diamond.

(B) Identification of the *O*-glucosylated form of ^{561}CQINIDDCQSQPCR^{574} from EGF repeat 14 in the control sample. The top panel shows a full MS spectrum of material eluting at 30.8 min. The ions labeled $[M+2H]^{2+}$ and $[M+3H]^{3+}$ match the predicted mass for doubly and triply charged forms of the glycopeptide, respectively. Other ions are from co-eluting material. CID fragmentation of the doubly charged form of this peptide, m/z 978.7 (top panel, $[M+2H]^{2+}$) resulted in the MS/MS spectrum shown in the bottom panel. The major product ion, m/z 897.5 ($[M+2H-Hex]^{2+}$), matches the mass for the unglycosylated peptide. The difference between the mass of the parent ion (m/z 978.5) and the product (m/z 897.5) matches the loss of a hexose during the fragmentation of a doubly charged peptide (81.2 Da X 2 = 162.4 Da). The presence of the *O*-glucose consensus sequence in the peptide indicates that the hexose is glucose. Confirmation of the m/z 897.5 species as ^{561}CQINIDDCQSQPCR^{574} comes from a number of y-ions in the MS/MS spectrum.

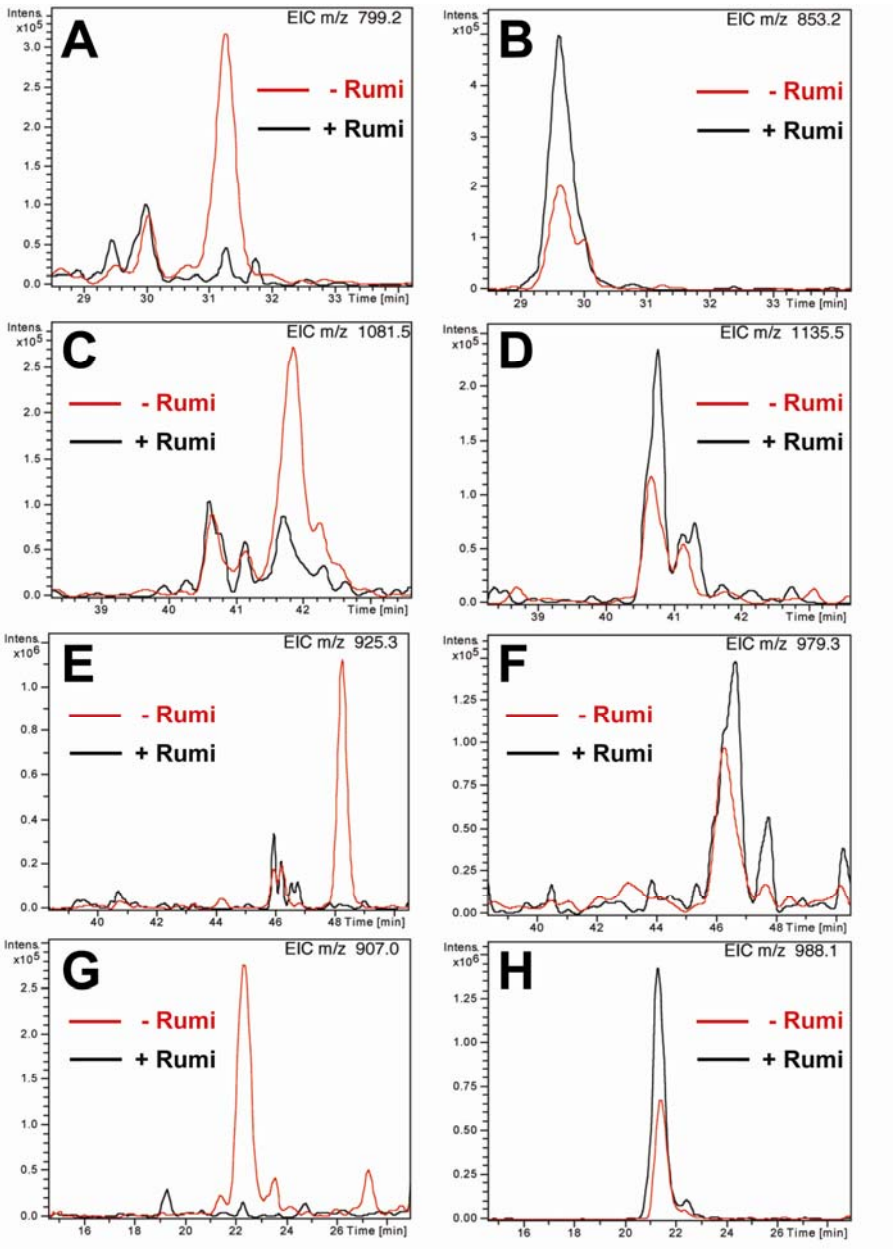

Figure S10. Knockdown of Rumi in S2 cells causes reduction in *O*-glucosylation of multiple EGF repeats in Notch

Tryptic peptides containing *O*-glucose consensus sequences from EGF repeats 16, 17, 19, and 35 were identified in the LC-MS data of EGF7-TM digests as described in Experimental Procedures and the legend to Figure S9. The chromatograms show elution of ions representing the unmodified peptides (A, C, E, G) or the corresponding glycopeptides (B, D, F, H). In each case, knockdown of Rumi (red lines) results in decreased levels of the glycopeptides and increased levels of the unmodified peptides compared to controls (black lines). The glycopeptides all elute slightly earlier than the corresponding unmodified peptide.

(A, B) Peptide from EGF repeat 16: ^{639}QINECESNPCQFDGHCQDR^{657}. Ion corresponding to triply charged (3+) form of this peptide (A, m/z 799.2) elutes at 31.2

min. Ion corresponding to the 3+ form of the glycopeptide (B, m/z 853.2) elutes at 29.6 min.
(C, D) Peptide from EGF repeat 17: ^{673}NCEVNVNECHSNPCNNGATCIDGINSYK^{700}. Ion corresponding to 3+ form of this peptide (C, m/z 1081.5) elutes at 41.8 min. Ion corresponding to the 3+ form of the glycopeptide (D, m/z 1135.5) elutes at 40.8 min.
(E, F) Peptide from EGF repeat 19: ^{744}GFYDAHCLSDVDECASNPCVNEGR^{762}. Ion corresponding to 3+ form of this peptide (E, m/z 925.3) elutes at 48.2 min. Ion corresponding to the 3+ form of the glycopeptide (F, m/z 979.3) elutes at 46.5 min.
(G, H) Peptide from EGF repeat 35: ^{1371}NCELSGQDCDSNPCR^{1385}. Ion corresponding to 2+ form of this peptide (G, m/z 907.0) elutes at 22.3 min. Ion corresponding to the 2+ form of the glycopeptide (H, m/z 988.1) elutes at 21.3 min.

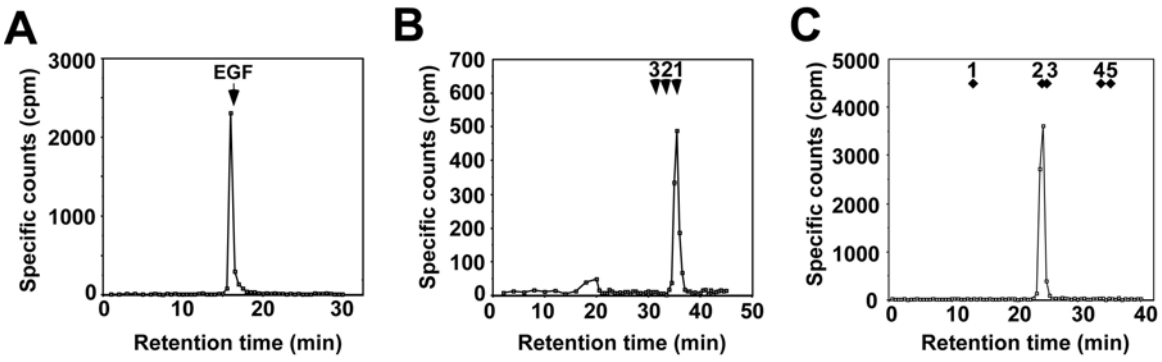

Figure S11. Product analysis demonstrates that Rumi adds a single *O*-glucose to factor VII EGF repeat
(A) The product of *O*-glucosyltransferase assays shown in Figure 6C contained factor VII EGF attached covalently to [^{3}H]glucose. The *arrow* shows the elution position of unmodified factor VII EGF. The *O*-[^{3}H]glucosylated EGF repeat elutes slightly earlier.
(B) The [^{3}H]glucose released from factor VII EGF by β-elimination was a monosaccharide. The elution positions of the sugar standards are indicated by *arrowheads* at the top of the graph (3: Gal-β1-4GlcNAc-β1-3-Fucitol, 2: Glc-β1-3-Fucitol, and 1: Fucitol).
(C) The [^{3}H]glucose released from factor VII EGF by β-elimination was in the form of glucitol. The elution positions of authentic standards are indicated by the *diamonds* at the top of the graph. 1: GalNitol, 2: Glucitol, 3: Galactitol, 4: Glucose, 5: Galactose.

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